Substitute Specification Attached

Our Ref.: B 2729 PCT

Method for producing plants having an increased tolerance against drought and/or fungal attack and/or increased salt concentrations and/or extreme temperature by the expression of plasmodesmata-localized proteins

Field of the Invention

The invention relates to the use of nucleic acids which code for a (poly)peptide with an intrinsic affinity to plasmodesmata, to the production of plants or parts thereof having an increased tolerance against drought and/or fungal infections and/or increased salt concentrations and/or extreme temperatures (heat/cold), and to corresponding methods. A plant, a plant tissue or a plant cell is advantageously transfected with the nucleic acid. The nucleic acid preferably codes for a virus-encoded transport protein which, in a particularly preferred embodiment, is a derivative of the pr17 protein with a hydrophilic N-terminal extension.

Background of the Invention

A number of documents are cited in this specification, the disclosure content of which is herewith incorporated by reference and the technical teaching of which can be applied within the meaning of the present invention.

One aim of classic plant breeding is the creation of productive varieties showing an increased tolerance against environmental factors or being resistant to stress factors. Those stress factors can be both of a biotic (insects, viruses, fungi etc.) and an abiotic nature (extreme temperatures, salt, drought). Whereas wildplants have adapted themselves to the extreme living conditions at stress-dominated habitats, drought, heat or salinity of the soil restrict the possibility of cultivating crop plants in such areas. On the other hand, agriculture also suffers heavy loss through abiotic stress at other habitats, as was shown in the USA in the year of drought in 1983: Almost half of the entire maize crop and a third of the expected soybean yield were destroyed as a consequence of persistent drought. All the cited abiotic stress factors impair the intercellular water balance. However, plants can adapt themselves to stress situations to a certain extent (Bohnert, (1995) Plant Cell **7**: 1099-1111). Proteins, for example, as well as metabolites of the plant metabolism like sugar alcohols, proline or glycine betaine have been identified as osmoregulators

accumulating as a result of abiotic stress. Based on that various strategies have been developed to produce transgenic plants having an increased tolerance against such factors or increased stress resistance by genetechnological modifications (article by Holmberg und Bülow (1998) Trends Plant Science **3**: 61-66). An example of plant proteins as anti-stress factors are the so-called LEA (late embryogenesis abundant)-proteins the increased expression of which correlates with physiological and environmental pressure and which perform a protective function for the plant in extreme stress situations (see for example Chandler (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. **45**: 113-141). The genetechnological modification of rice with the help of the barley-LEA-gene HVA1 indeed resulted in an increased tolerance against drought and salt (Xu (1996) Plant Physiology **110**: 249-257). Further tests relating the expression of a LEA-genes in a heterologous system could not support these results (Iturriaga (1992) Plant Mol. Biol. **20**: 555-558).

These plant metabolites identified as anti-stress factors or osmoprotectors include glycine betaine the effectiveness of which has been proved for example by maize plants (Saneoka, (1995) Plant Pysiol. **107**: 631-638). The synthesis of the glycine betaine in plant-chloroplasts is supported by the betaine aldehyde dehydrogenase (BADH) (Rhodes, (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. **44**: 357-384). Transgenic tobacco plants expressing a bacterial BADH (Holmstrom, (1994) Plant. J. **6**: 749-758) or a vegetable BADH (Rathinasabapathi, (1994) Planta **193**: 155-162) showed the expected resistance to betaine aldehyde by conversion to glycine-betaine in amounts which are measured in plants exposed to stress. Nothing, however, was reported on an increased stress tolerance of these plants.

Obviously, derivatives of sugar like sugar alcohols or fructans are also produced and accumulated to a higher degree as a stress response by the plant. The increase of the mannitol level in transgenic tobacco plants by expression of a bacterial mannitol-1-phosphate-dehydrogenase resulted in an increased salt tolerance (Tarczynski, (1993) Science **259**: 508-510). Equally, transgenic tobacco plants with a higher fructan level showed an increased resistance to drought compared to control plants (Pilon-Smith, (1995) Plant Physiol. **107**: 125-130).

Summary of the Invention

All the genetechnological methods mentioned up to now have an increased synthesis of osmoprotectors in the plant in common, by which the normal growth of the plant can be impaired (see Rathinasabapathi, op. cit.). In other cases bacterial genes are expressed in the plant, which is not necessarily accompanied by an optimal expression and therefore not by a suboptimal stress mastering. Consequently, the

technical underlying of the present invention was to provide a method for creating plants with an increased stress resistance which still show an essentially normal growth. This stress resistance (or tolerance) should preferably refer to both biotic and abiotic stress factors. This technical problem has been solved by providing the embodiments characterised in the claims.

Thus, the invention relates to the use of a nucleic acid which codes for a (poly)peptide with an intrinsic affinity to plasmodesmata to the production of plants or parts thereof having an increased tolerance against drought and/or fungal infections and/or increased salt concentrations and/or extreme temperatures, i.e. cold and/or heat. According to the invention, a plant, a plant tissue or a plant cell is usually transfected with such a nucleic acid according to standard methods.
All higher plants are characterised by their capability to create photosynthesis of sugars and their derivatives which can serve osmoprotectors in stress situations, as already mentioned above. The intercellular concentration of sugar and sugar derivatives - above all in the leaves for the protection of the photosyntheticallly-active chloroplasts – was solved, according to the invention, in an unexpectedly simple way by the measure mentioned above. Of a particular advantage is furthermore, apart from the fact that these measures can easily be worked out in a technical way by a skilled person that, according to the invention, the stress resistance can be increased with a mechanism which is valid in many and possibly even in all plants. Surprisingly, the plant tolerance against abiotic as well as against biotic factors could be increased with the method of the invention.

Brief Description of the Drawings

Figure 1
Production of the plasmid p17N. By specific mutagenesis the two AUG-codons of the wildtype pr17-gene were mutated to ACG and a translation initiation codon was inserted into the polylinker sequence.

Figure 2
Nucleotide and amino acid sequence of the mutated pr17N-gene or –protein (SEQ ID NOS 2 & 3).

Figure 3
Result of the resistance test #1 (in the green-house) with 5 plants each of the initial variety Linda (L) as well as the pr17N-transgenic lines L4, L6, L7 and L8.
A. Overall view of the test. B. View of one plant each per line.

Figure 4
Result of the resistance test #2 (in the phytochamber) with 6 plants each of the initial type Linda (L) as well as of the pr17N-transgenic lines L4, L6, L7 and L8.
A. Partial view of the overall test. B. View of selected plants.

Figure 5
Bonitation of the infestation of leaf disks in the laboratory test with *P.infestans* race 1-11 (test #2) after 9, 10 and 13 days after the infection (dpi).

Figure 6
Cumulative depiction of two tests concerning the infection of potato leaf disks with *P. infestans* race 1-11.

Figure 7
Habitus of the non-transgenic potato variety Linda (L) and of the 4 transgenic lines L4, L6, L7 and L8 after 5 weeks at 100 mM NaCl. Individual plants of the partial test (A) were described for a better depiction in (B). Concerning the initial variety Linda the lower leaves died off.

Figure 8
Habitus of individual plants of the non-transgenic potato variety Linda (L) and of the 4 transgenic lines L4, L6, L7 and L8 after 5 weeks with 100 mM NaCl. Concerning variety Linda there can be still recognised residues of the died off lower leaves as well as modifications of the stalk.

Detailed Description of the Invention

The term "increased salt concentration" refers to salt concentration in the soil leading to an increased ionic concentration in the plant which in turn leads to reduced growth. The absolute salt concentrations in the soil which have to be regarded as increased are different for different plants. They can, however, be determined by a skilled person according to standard methods, like for example by means of the disclosure content of Greenway and Munns (1980) Ann. Rev. Plant. Physiol. **31**: 149-190.

For the expression of the nucleic acid encoding a (poly)peptide with an intrinsic affinity to plasmodesmata in plant cells it is connected with regulatory sequences ensuring the transcription in plant cells. Among these are above all promoters. Generally, each promoter which is active in plant cells can be used for the expression.

The promoter can be either of a nature so that the expression takes place in a constitutive way or only in a certain kind of tissue, at a certain time of the plant development or at a point in time determined by external influences.

Concerning the plant the promoter can either be homologous or heterologous. Useful promoters are for instance the promoter of the 35S RNA of the Cauliflower Mosaic Virus (CaMV) and the ubiquitin-promoter of maize for a constitutive expression. Furthermore, there is usually a transcription-termination-sequence which serves the correct termination of the transcription and which can as well serve the addition of a poly-A tail to the transcript which performs a function regarding the stabilisation of the transcripts. Such elements like e.g. the terminator of the octopine synthetase gene of agrobacteria are described in the literature (see Gielen, EMBO J. 8 (1989), 23-29) and are interchangeable at will. In a preferred embodiment the promoter is the 35S CaMV promoter.

Apart from the nucleic acid which encodes a (poly)peptide with an intrinsic affinity to plasmodesmata the plant which is used or produced in any of the methods of the invention can contain further recombinant DNA-molecules which can, for example, be used for plant protection or quality enhancement of the plant or the harvest product thereof. Examples of plant protection measures include: (i) tolerance against herbicides (DE-A-3701623; Stalker (1988) Science 242, 419), (ii) resistance to insects (Vaek (1987) Plant Cell 5, 159-169), (iii) resistance to viruses (Powell (1986) Science 232, 738-743) and (iv) resistance to ozone (Van Camp (1994) BioTech. 12, 165-168). Examples of quality enhancement are: (i) enhancement of imperishability of fruits (Oeller (1991) Science 254, 437-439), (ii) enhancement of the starch production in potato tubers (Stark (1992) Science 242, 419), (iii) modification of the composition of starch- (Visser (1991) Mol. Gen. Genet. 225, 289-296) and lipid compositions (Voelker (1992) Sciene 257, 72-74) and (iv) production of polymers foreign to plant (Porier (1992) Science 256, 520-523).

The availability of appropriate transformation systems is a prerequisite for the production of transgenic plants is. At the moment several methods are available for the transformation. The method for the transformation of dicotyledonous plants used most frequently is the gene transfer mediated by agrobacteria. In this connection the natural ability of the soil bacterium to integrate genetic material into the plant genome is used. Further appropriate methods are, for example, protoplast transformation by polyethyleneglycol-induced DNA-absorption, electroporation, sonication or microinjection as well as the transformation of intact cells or tissues through micro- or macroinjection in tissues or embryos, tissue electroporation, incubation of dry

embryos in a solution containing DNA, vacuum infiltration of seeds and biolistic gene transfer (for an overall view: see e.g. Potrykus, Physiol. Plant (1990), 269-273 and Christou (1996) Trends in Plant Science 1, 423-431).

Whereas the transformation of dicotyledonous plants via Ti-plasmid vector systems with the help of Agrobacterium tumefaciens is well established, more recent works indicate that monocotyledonous plants are indeed available for the transformation through vectors based on the Agrobacterium (Chan, Plant Mol. Biol. 22 (1993), 491-506; Hiei, Plant J. 6 (1994), 271-282; Bytebier, Proc. Natl. Acad. Sci. USA 84 (1987), 5345-5349; Raineri, Bio/Technology 8 (1990), 33-38; Gould, Plant Physiol 95 (1991), 426-434; Mooney, Plant, Cell Tiss. & Org. Cult. 25 (1991), 209-218; Li, Plant Mol. Biol. 20 (1992), 1037-1048). The methods for the introduction of foreign DNA by means of the biolistic method or by protoplast transformation are known (see e.g. Christou (1996) Trends in Plant Science 1, 423-431; Willmitzer, L., 1993 Transgenic plants. In: Biotechnology, A Multi-Volume Comprehensive Treatise (H.J. Rehm, G. Reed, A. Pühler, P. Stadler, eds.) Vol. 2, 627-659, VCH Weinheim-New York-Basel-Cambridge). In the past it was possible to establish three of the above-mentioned transformation systems for various cereals: the electroporation of tissue, the transformation of protoplasts and the DNA-transfer through particle bombardment into regenerable tissues and cells (for an overall view: Jähne, Euphytica 85 (1995), 35 - 44). The transformation of wheat is described several times in the literature (for an overall view: Maheshwari, Critical Reviews in Plant Science 14 (2) (1995), 149 – 178). The skilled person can make use of the markers for the selection of transformed plant cells, plant tissues and plants. The transgenic plant cells can be regenerated into whole plants by means of techniques known to the skilled person. The skilled person can also make use of e.g. molecular biological methods like PCR in order to identify these plants. On the other hand, the skilled person can, of course, e.g. after self-fertilisation or back-crossing against the parent, also place seeds of such plants on selective media and by means of the germinative capacity of these seeds or the survival of the plants at a later stage of the development (depending on the chosen promoter) he can find out, whether the plants are transgenic or not. Basically, transgenic plants can be plants of any plant species, i.e. they can be either monocotyledonous or dicotyledonous plants.

As, regardless of the transformation method, only few cells have the desired characteristics a selectable marker is integrated into the plant genome in addition to the target gene in a conventional way. At the moment, above all genes which transmit tolerance towards herbicides or antibiotics are used for the selection of transformed plant cells. Appropriate resistance genes are, for example, the bar-gene

of Streptomyces hygroscopicus which mediates resistance towards the total herbicide Phosphinothricin (De Block (1987) EMBO J. 6, 2513-2518) or the nptII-gene from the transposon Tn5 of Escherichia coli which leads to resistance towards the antibiotic kanamycin (Herrera-Estrella (1983) EMBO J. 2, 987-995). Further selection systems are e.g. expression of a mannose-6-phosphate-isomerase and positive selection on nutritive media containing mannose (WO 94/20627). A further method makes use of the ability of a deaminase of Aspergillus terreus to detoxicate the insecticide blasticidin S (Tamura (1995) Biosci. Biotechnol. Biochem. 59, 2336-2338). Of course, the skilled person knows that the selection marker does not necessarily need to be present in the vector containing the recombinant DNA-molecule, but it can also be co-transformed with the vector (Lyznik (1989) Plant Mol. Biol. 13, 151-161; Peng (1995) Plant Mol. Biol. 27, 91-104). This possibility is, for example, useful if no physical linkage of the marker gene and the information which is to be transformed is desired.

In a preferred embodiment of the use according to the invention a plant is additionally regenerated from the transfected plant cell. The regeneration of the plant can take place according to methods which are conventional and known by the skilled person.

In an especially preferred embodiment according to the use of the invention further plants or plant cells are produced additionally of the regenerated plant after the regeneration step.

In a further preferred embodiment according to the use of the invention the (poly)peptide is a virus-encoded transport protein.

The expression of plant viral proteins which take part in the transport of viral information from cell to cell (transport proteins) also influence the transport or metabolism of starch, sugars and sugar derivatives in such a way that they accumulate to values exceeding the normal values during the light period in the photosynthetically active leaves of the plant (see e.g. Lucas 1993) Planta **190**: 88-96; Olesinski (1995), Planta **197**: 118-126; Olesinski, (1996) Plant Physiol. **111**: 541-550; Herbers (1997), Plant J. **12**: 1045-1056; Almon (1997), Plant Physiol.**115**: 1599-1607). The tolerance increase in of plants towards the above-mentioned environmental factors through expression of such proteins with an intrinsic affinity to plasmodesmata, the communicating galleries of adjoining cells in plants according to this preferred embodiment of the invention could not be deduced easily from the prior of the art. The normal function of virus-encoded transport proteins (TP) is to ensure

the transport of the genetic information of a virus from cell to cell and thus to enable the spread of a virus from the original infection place to the whole plant.

In a particularly preferred embodiment of the use according to the invention the virus-encoded transport protein is the potato leaf roll virus-(PLRV) transport protein pr17 or a derivative thereof.
As illustrated in the examples the TP of the potato leaf roll-virus (PLRV) as well as the cultivated plant potato were chosen as a model system. The PLRV-TP which describes a characteristic of a particularly preferred embodiment of the method of the invention is a 17 kDa large protein (pr17) which is responsible for the transfer of the genomic RNA from cell to cell. It is encoded by means of the open reading frame (ORF) ORF4; this gene lies in the ORF3, the gene for the viral capsid protein CP, however, in a different reading frame. The protein possesses an aminoterminal domain for the formation of homopolymers (Tacke (1993), Virology **197**: 274-282) and a carboxyterminal domain for binding single-stranded nucleic acids (Tacke (1991), J. Gen. Virol. **72**: 2035-2038). This protein which is present in *in planta* phosphorylated form (Tacke (1993), op. cit.; Sokolova (1997), FEBS Lett. **400**: 201-205) is expressed seven times more strongly than the viral-coat-protein gene (Tacke (1990), J. Gen. Virol. **71**: 2265-2272). Both in PLRV-infected and pr17-transgene potato plants the pr17 is predominantly located in the plasmodesmata between sieve element and companion cell of the phloem (Schmitz (1997), Virology **235**: 311-322), to which the virus is limited during its reproduction in the plant. Expression of a mutated pr17-protein in transgenic potato plants leads to broad-spectrum resistance against the most important potato viruses (Tacke (1996), Nature Biotechnology **14**: 1597-1601). At the same time, however, in the course of this invention it was observed that the expression of WT and mutated PLRV-TPs in transgenic potato- and tobacco plants leads to an increase in the intracellular concentrations of sugars (sucrose, fructose, glucose) and derivatives of sugar like starch.

In a further preferred embodiment of the use according to the invention the derivative is a pr17 protein with a hydrophilic N-terminal extension.
Particularly good results in the sense of the invention were found when a variant of the pr17-gene was used which carries a N-terminal extension (pr17-N). In an embodiment the polylinker (multiple cloning site; MCS) of the Bluescript-vector was fused in a translational way to the 5'-end of the pr17-WT-gene and the first two AUG-translation codons of pr17 in ACG-codons were mutated by means of selective mutagenesis and an AUG-translation start codon was inserted into the polylinker sequence (Figur 1). This modification results in the expression of a derivative (pr17-N) of the pr17-WT-proteins with a hydrophilic extension through the sequ nce

MAELGSGSELHRGGGRSRTS (SEQ ID NO: 1) at the amino terminus (Tacke et al., 1996, op. cit.; Figure 2). In the green-house test such transgenic potato plants show broad-spectrum resistance against potato viruses PLRV, PVY and PVX as well as an increased concentration of sugar and sugar derivatives.
For the expression in plants this gene was brought under transcriptional control of the 35S-promoter and –terminator of the cauliflower-mosaic-virus (CaMV) in the vector pRT103 (Töpfer (1987), Nucleic Acids Res. **15**: 5890) and this transcription unit (Figure 1) was then integrated into the binary plant transformation vector pBIN19 (Bevan (1984), Nucleic Acids Res. **12**: 8711-8721). This vector was transferred to the *Agrobacterium tumefaciens* stem LBA4404 (pAL4404) (Hoekema (1983), Nature **303**: 179-180) which was used for the transformation of *Solanum tuberosum* Var. Linda. Four (L4, L6, L7 and L8; see also Tacke (1996), op. cit.) of the independent transgenic potato lines as well as the initial potato variety Linda were chosen for further tests concerning induced tolerance.

As already mentioned above the hydrophilic extension comprises the amino acid sequence MAELGSGSELHRGGGRSRTS (SEQ ID NO: 1) in a particularly preferred embodiment of the use according to the invention.

In another embodiment of the use according to the invention the plant, the plant tissue or the plant cells stem from the potato, from tobacco, from cereals or vegetables or are potatoes, tobacco plants, cereal plants or vegetable plants.

In a further preferred embodiment of the use according to the invention the increase in the tolerance of the plants against fungal infections is a tolerance against infections with Phytophthora infestans.
As a surprising result according to this preferred embodiment it was found that transgenic lines received by the method according to the invention also distinguish themselves by a statistically significant tolerance against *Phytophthora infestans*, the pathogen of late blight of potato.

The invention further relates to the production of plants or parts thereof with an increased tolerance against drought and/or fungal infections and/or increased salt concentrations and/or extreme temperature (heat, cold), wherein

(a) a plant, a plant tissue or a plant cell is transfected with a nucleic acid coding for a (poly)peptide with an intrinsic affinity to plasmodesmata.

Additionally, in a preferred embodiment of the method according to the invention

(b) a plant is regenerated from the transfected plant cell.

In a particularly preferred embodiment of the method according to the invention

(c) further plants or plant cells are produced from the plant gained in (b) subsequent to step (b).

In a further preferred embodiment of the method according to the invention, the polypeptide is a virus-encoded transport protein.

In a particularly preferred embodiment of the method according to the invention the virus-encoded transport protein is the potato leaf roll virus-(PLRV) transport protein pr17 or a derivative thereof.

In a further preferred embodiment of the method according to the invention the derivative is a pr17 protein with a hydrophilic N-terminal extension.

As already mentioned above in a particularly preferred embodiment of the method according to the invention the hydrophilic extension comprises the amino acid sequence MAELGSGSELHRGGGRSRTS (SEQ ID NO: 1).

In another embodiment of the method according to the invention the plant, the plant tissue or the plant cells stem from potato, from tobacco, from cereals or vegetables or are potatoes, tobacco plants, cereal plants or vegetable plants.

In a further preferred embodiment of the method according to the invention the increase in tolerance of the plant against fungal infections is a tolerance against infections with Phytophthora infestans.

The Examples explain the invention.

Example 1: Production of the plasmid p17N

A modification at the 5'-end of the pr17-gene (ORF4) was achieved by translational fusion of the multiple cloning site of the Bluescript-vector, insertion of an optimised translation initiation codon as well as mutation of the two pr17-WT AUG initiation codon to ACG (Figure 1). This modification results in the expression of a derivative (pr17-N) of the pr17-WT-protein with a hydrophilic extension through the sequence MAELGSGSELHRGGGRSRTS (SEQ ID NO: 1) at the amino terminus (Tacke (1996), op. cit.; Figure 2). The production of the plasmid p17N is described in Schmitz (1996), Nucleic Acids Res. **24**: 257-263 (therein named p17/NIII).

Example 2: Introduction of the T-DNA in the recipient organism

After the transformation of *E. coli* S17-1 cells and mating with the *A. tumefaciens* stem LBA 4404 (pAL 4404) (Hoekema *et al.*, 1983) agrobacteria carrying the plasmid p17N were used for plant transformation. Leaves of *S. tuberosum var.* Linda of the sterile culture were cut at the basis and incubated for 10 min in liquid MS-medium with an agrobacteria culture grown over night. After two days of incubation on solid MS-medium the leaves were washed and placed on selection-, regeneration-medium. This consisted of MS-medium complemented with 0.02 mg/l naphtyl acetic acid, 0.02 mg/l gibberellin acid, 2 mg/l zeatinriboside, 500 mg/l Claforan and 100 mg/l kanamycin sulfate. Sprouts developed after 6-8 weeks and were transplanted on MS-medium with 250 mg/l claforan and 150 mg/l canamycinsulfate for the root formation.

Example 3: Characterisation of the transgenic lines obtained

The expression of the N-terminal modified PLRV 17K TP was detected in Western blot with the help of 17K-specific antiserums as described in Tacke et. al., 1996 (op. cit.). For this purpose protein extracts were produced of leaf material, the proteins were separated on a 12.5% polyacrylamide gel, transferred to nitrocellulose membranes and incubated with a 17K-specific antibody. The evidence of bound antibodies was carried out according to reliable methods by incubation with sheep-anti-rabbit-IgG/peroxidase conjugate and the peroxidase substrate of the ECL-kits (Amersham).

Example 4: Resistance test with *Phytophthora infestans*

For the determination of the quantitative resistance infestation tests were carried out in the laboratory using the pathogen of late blight of potato, *Phytophthora infestans,*. The 1-11 race of the pathogen was used as inoculum (for the preservation this race is cultivated on leaf material of the potato variety Désireé). For this purpose small potato leaves were inoculated with the pathogen in an irrigation box (Gieffers (1989), J. Phytopathology **126:** 115-132) which enables a permanent water supply, and were placed at 10°C. The light supply was effected by 10 lamps of the type Osram, L16, W/25 Weiß Universal for 16 h daily with a medium photoperiodically active radiation of about 100 $\mu mol\ s^{-1}m^{-2}$. The sporangia which were formed after 8-10 days were washed off from the small leaves with water. The share of living sporangia was determined by the staining method according to Behr (Behr (1955) Zentralblatt für Bakt. Etc. II **108**: 23/24, 641-656). For infestation tests a suspension with a sporangia density of $10^4\ ml^{-1}$ was used.

Following the test according to Hodgson (Hodgson (1961), American Potato Journal **38**: 259-264) a new testing method was developed. By means of a punching machine which was developed for the test, leaf disks of a diameter of 20 mm were produced. The punching method works with low pressure to avoid crushing of the leaf edges and necrosis as well as bacterial decomposition.

The leaf disks are placed in irrigation boxes (Gieffers et al., op. cit.) on filtering paper with the bottom side up. The permanent water film on the filtering paper provides the leaf disks with sufficient water. For inoculation a drop with a sporangia suspension (200 sporangia/20 µl) is pipetted onto the middle of the leaf disk.

The inoculated leaf disks are incubated at a permanent temperature of 10°C and under the above-mentioned light conditions. Under these conditions the infectious zoosporangia hatch. After about 6 days the first sporangia developed, the infestation bonitation is effected after 8 to 10 days.

The leaf disk infestation, recognisable by sporangia turf-forming and leaf tissue decomposition, is assessed in percentage.

An important prerequisite for the test is that the potato plants which are to be tested are cultivated under the same conditions and that the leaf disks are gained from small leaves of the same leaf storey and the same leaf position.

In this way the quantitative infestation of green-house- and open land material can be tested. The degree of infestation decides on the quantitative degree of resistance.

Example 5: Induced tolerance against drought as an example of increased tolerance against abiotic stress

5 or 6 plants each of the transgenic lines L4, L6, L7 and L8 as well as the initial variety Linda were kept under drought stress for 8 weeks in the 6-8 leaf-stage with the plant being irrigated after 3 and 6 weeks. In this connection the transgenic plants showed a clearly increased tolerance against water stress in two independent tests as can be seen in Table 1 and the Figures 3 and 4. Table 1: Analysis of the potato lines L4, L6, L7 and L8 as well as the initial variety Linda after 8 weeks of water stress.

Line / type # surviving plants / # of the examined plant

	Test 1**	test 2***
Linda	1*/5	1*/6
L4	4/5	6/6
L6	5/5	6/6
L7	5/5	6/6
L8	5/5	5/6

*The surviving plant develops a new sprout from the tuber; all the original parts of the plant are dead in contrast to the surviving plants of the transgenic lines L4, L6, L7 and L8.
** This test was carried out under controlled conditions in the green-house.
***This test was carried out under controlled conditions in a phytochamber.

Example 6: Induced tolerance against *Phytophthora infestans* as an example of increased tolerance against fungal infection

Two independent infestation tests with *P. infestans* were carried out with the 1-11 races on leaf disks of green-house material. In this connection 8 leaf disks of 8 plants each per transgenic lines and initial variety Linda were used for infection in the laboratory test. The inoculum contained 300 sporangia per 20 µl water; the incubation of leaf disks was effected at 10°C and bonitations were carried out after 9, 10 and 13 days after the infection (dpi). All the 4 transgenic lines showed a significant lower infestation of up to 10 dpi compared to *P. infestans*. After that the infestation at L7 and L8 increased rapidly whereas L6 and particularly L4 maintained their relatively low degree of infestation (Figure 5). On average of both tests there is a significant difference regarding the resistance conduct of the 4 tested transgenic Linda-lines compared to the non-transgenic initial type Linda (Figure 6).

Example 7: Induced tolerance against salt as a further example of increased tolerance against abiotic stress

In a series of tests of 4 plants each of the lines L4, L6, L7 and L8 as well as the non-transgenic initial variety Linda (L) the potato plants were kept in granulate for 5

weeks under salt stress, wherein salt in the form of NaCl in concentrations of 10, 25, 50, 75, 100, 200, 400 and 1000 mM was contained in the irrigation water. The highest concentration of 1000 mM lead to heavy damage among all plants after 2 weeks and these plants were dead after 5 weeks, whereas the transgenic lines, however, not variety Linda, survived a salt concentration of 400 mM despite heavy damage. As depicted in Figures 7 and 8 for the irrigation with 100 mM NaCl after 5 weeks, all transgenic as well as all non-transgenic plants had a similar phenotype (the somewhat smaller growth height of the transgenic lines as depicted in Figure 7B is due to the genetic modification not to the salt stress): Concerning the non-transgenic initial variety Linda the lower leaves died off and the stalk shows characteristic pathological modifications (strong stenosis, brown coloring), as is also depicted in Figure 8, whereas regarding all of the transgenic lines the stalks are as green as before (Figure7, 8) and the leaves did not die (Figure 7A,B). At most, there are a few isolated cases of necrosis on the lower leaves (Figure 8). Despite the salt stress all plants develop blossoms and tuber formation can be observed. (Figure 8).